Multicellular Redox Regulation in an Early-Evolving Animal Treated with Glutathione

Joseph F. Doolen
Gabrielle C. Geddes
Neil W. Blackstone*
Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115

Accepted 12/21/2006; Electronically Published 2/8/2007

ABSTRACT

Redox signaling has emerged as a unifying theme in many seemingly disparate disciplines. Such signaling has been widely studied in bacteria and eukaryotic organelles and is often mediated by reactive oxygen species (ROS). In this context, reduced glutathione (GSH) acts as an important intracellular antioxidant, diminishing ROS and potentially affecting redox signaling. Complementing this cell-level perspective, colonial hydroids can be a useful model for understanding organism-level redox signaling. These simple, early-evolving animals consist of feeding polyps connected by tubelike stolons. Colonies treated exogenously with GSH or reduced glutathione ethyl ester (GEE) were expected to show a morphological change to sheetlike growth typical of low levels of ROS. Contrary to expectations, diminished stolon branching and polyp initiation was observed. Such runnerlike growth is associated with higher levels of ROS, and surprisingly, such higher levels were found in GSH- and GEE-treated colonies. Further investigations show that GSH triggered a feeding response in hydroid polyps, increasing oxygen uptake but at the same time relaxing mitochondrion-rich contractile regions at the base of polyps. Diminished gastrovascular flow and increased emissions of mitochondrial ROS also correlated with the observed runnerlike growth. In contrast to cell-level, "bottom-up" views of redox signaling, here the phenotype may arise from a "top-down" interaction of mitochondrion-rich regions and organism-level physiology. Such multicellular redox regulation may commonly occur in other animals as well.

* Corresponding author; e-mail: neilb@niu.edu.

Physiological and Biochemical Zoology 80(3):317–325. 2007. 1522-2152/2007/8003-6832$15.00

Introduction

Signaling pathways mediated by the chemistry of oxidation and reduction ("redox signaling") have emerged as a unifying theme in many seemingly disparate disciplines, ranging from cell death to aging and biological time (Balaban et al. 2005; Gillette and Sejnowski 2005; Guarente and Picard 2005). Redox chemistry involves the transfer of electrons and hydrogen atoms and is central to energy conversion in metabolism. Typically in metabolic processes, environmental oxidants and reductants are linked by a series of electron carriers operating close to their midpoint potentials. Perturbations of these redox potentials by environmental changes in electron sources and sinks lead to measurable signals (Pfannschmidt et al. 1999; Allen et al. 2005). These signals can be transduced to proteins, the primary gene products, by various means, including reactive oxygen species (ROS; Coffman et al. 2004; Nedelcu et al. 2004; Haridas et al. 2005). Broadly defined, ROS are partially reduced forms of oxygen such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$). ROS can reversibly interact with proteins in a number of ways, such as altering iron-sulfur clusters (Armstrong et al. 2004) or oxidizing cysteine and histidine residues (van Montfort et al. 2003; Lee and Helmann 2006). The conformation and activity of such proteins can thus be reversibly affected. If the target proteins regulate biochemical, physiological, or developmental pathways, a rapid and precise response to metabolic perturbations can thus ensue.

Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is a common peptide found in most organisms. It consists of three amino acids: glutamine, glycine, and cysteine. Often found in millimolar concentrations, it is the most abundant nonprotein thiol in many cells and serves as a source for reducing equivalents in many enzymatic and nonenzymatic reactions. This results in diverse functions for GSH: it is a principal intracellular antioxidant, it maintains the thiol redox status of the cell, it transports and stores cysteine, it modulates cell proliferation and differentiation via its effects on protein and DNA synthesis/function, and it regulates signal transduction for multicellular organisms (Butterfield et al. 2002; Jefferies et al. 2003; Filomeni et al. 2005). When added exogenously, GSH enters cells only to a limited extent. The ethyl ester tag of glutathione ethyl ester (GEE) allows the import of the GSH molecule into the cell via the reduction of GEE by esterases into GSH and ethanol (Takano et al. 2002).

Complementing this cell-level perspective, simple early-evolving animals, such as cnidarians, can be useful models for

understanding organism-level redox signaling. Particularly well studied in this regard are colonial hydroids such as *Podocoryna* (= *Podocoryne*) *carnea*, which consist of polyps connected by tubelike stolons. Polyps feed and then pump gastrovascular fluid throughout the colony. Mitochondrion-rich epitheliomuscular cells (EMCs) facilitate gastrovascular flow. In *P. carnea*, these mitochondrion-rich cells are concentrated at the polyp base, and their hypothesized function is to pull open the gastrovascular lumen connecting the polyp to the stolon. Strong exertions by these cells thus correlate with high rates of gastrovascular flow into and out of the polyp (Blackstone 2001). Such high levels of metabolic demand produce relatively oxidized redox states and low levels of ROS emissions from these mitochondrion-rich cells (Blackstone 2001, 2003). When these cells relax, on the other hand, the polyp-stolon junction likely closes. Generally, this junction is closed before, while, and for a short time after polyps feed (Dudgeon et al. 1999). During these times, mitochondrion-rich cells exhibit relatively reduced redox states and higher levels of ROS emissions. In general, colony growth and form is sensitive to the redox state and ROS emissions of these cells (Blackstone et al. 2004, 2005*a*, 2005*b*). Compared with colonies that are relatively oxidized with lower levels of ROS, colonies with relatively reduced redox states and higher levels of ROS exhibit higher rates of outward growth and lower rates of stolon branching and polyp initiation ("runnerlike" growth).

From a cell-level, "bottom-up" perspective, treatments with antioxidants to some extent allow decoupling mitochondrial redox state from ROS levels. In other words, treatment with antioxidants typically will diminish ROS even if mitochondrial electron carriers are highly reduced and continue to produce quantities of ROS. Independent of manipulations of mitochondrial redox state (e.g., Blackstone 2003), the extent to which ROS mediate redox signaling can thus be assessed (e.g., using vitamin C; Blackstone et al. 2005*a*). In this context, studies of the effects of GSH and GEE on colonial hydroids were undertaken. The initially unexpected results (that these antioxidants increase mitochondrial ROS and correlated runnerlike growth) highlighted the complexity of organism-level responses and led to further experiments with GSH and measures of redox state, gastrovascular flow, and oxygen uptake. Because the data appear inconsistent with a cell-level perspective, an organismal or "multicellular" view of redox regulation is developed and illustrated. Multicellular redox regulation may generally illuminate "top-down" redox signaling mechanisms in animals.

Material and Methods

Study Animals and Culture Conditions

Colonies of the P3 clone of *Podocoryna carnea* were cultured using standard methods (e.g., Blackstone 1999). Colonies were grown on glass cover slips and confined to one side of the cover slips by daily scraping with a razor blade. Each experiment included a control group explanted from the same colony, because these organisms are sensitive to subtle environmental and epigenetic effects (Ponczek and Blackstone 2001). All experiments were carried out at ~20.5°C. Any study of the effects of GSH and GEE on hydroids or other cnidarians should be prefaced by mention of the well-studied GSH-mediated feeding response in these organisms. This response may occur generally in cnidarians and has been particularly well studied in *Hydra* species. In the presence of 5–10 μmol L^{-1} of GSH, *Hydra* polyps writhe their hypostomes and tentacles and open their mouths (Lenhoff 1961). Inasmuch as prey wounded by cnidarian nematocysts may release GSH, this response is thought to be adaptive. At the outset of this study (and from a cell-level perspective), it was expected that the antioxidant properties of GSH and GEE could be studied independently of this feeding response; this issue is returned to below in "Discussion."

Comparisons of Colony Growth and Development

Colonies were treated in finger bowls (GSH; 65 μmol L^{-1}; concentrations as high as 100 μmol L^{-1} were tested) or Petri dishes (GEE; 1 mmol L^{-1}; note the considerably higher concentration than that of GSH; lower concentrations were found to have no detectable effect) for ~4–6 h d^{-1}. In separate experiments over the course of a year, seven colonies were treated with GSH or GEE, and seven control colonies were used. Two experiments were done with GSH; in one, only four controls were imaged because the cover slips for the others were accidentally broken; this experiment is referred to in the results as "GSH2." Each colony was imaged when nearly covering the surface of the 18-mm cover slip (up to 60 d after explanting). Operationally, the time of imaging was defined as the growth stage in which the colony exhibited multiple stolons contacting the edge in at least three of the four quadrants of the cover slip. Images were processed to facilitate automatic measurement using Image-Pro Plus software (Blackstone et al. 2004). Processed images were checked against the original images to insure accuracy. Each processed image was measured in Image-Pro Plus software for areas of polyps and unencrusted coverslip (Blackstone et al. 2004). Total colony area was measured as well. Using PC-SAS software, data for areas, polyp areas, and unencrusted areas were compared between treated colonies and controls using ANOVA. Data were natural-log transformed when necessary to meet the assumptions of parametric statistics.

Comparisons of ROS, Redox State, and Gastrovascular Flow

Hydrogen peroxide represents a major component of ROS under physiological conditions (Chance et al. 1979), and measures of H_2O_2 were taken using 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; Nishikawa et al. 2000; Pei et al. 2000). The activation of H_2DCF can be relatively specific for the detection of H_2O_2; alternatively, this can be regarded as a general, semi-

quantitative assay of ROS (Finkel 2001). Twenty-four hours after feeding, colonies were incubated in GSH and GEE as above. Within 1 h, H_2DCFDA was added to a concentration of 10 μmol L^{-1}, and colonies were incubated for an additional hour in the dark before measurement. Control colonies were similarly treated. Using a Hamamatsu Orca-100 cooled-CCD camera and a Zeiss Axiovert 135, ROS were imaged for a ~50 × 150 μm contractile region at the base of three polyps per colony (excitation 450–490 nm, emission 515–565 nm).

Images thus obtained were analyzed using Image-Pro Plus software. In such images, fluorescence is visible from many ~10-μm^2-sized clusters of mitochondria from EMCs at polyp-stolon junctions (Blackstone et al. 2004; such mitochondrion-rich cells have not been found elsewhere in the colony). The luminance and area for each of these fluorescent objects were measured and adjusted for the background luminance by subtraction. Typically, these methods lead to a trade-off between area and luminance: the larger the area of luminance identified by the software, the dimmer the relative luminance. Measures were analyzed by a nested ANOVA with mitochondrial clusters nested within polyps, polyps nested within clonal replicates, and replicates nested within treatments (Blackstone et al. 2004).

The similar effects of GSH and GEE treatments on colony growth, development, and ROS levels (see "Results") suggest that both have the same extracellular effect and that GSH is considerably more potent in triggering this effect. Therefore, subsequent experimentation focused on GSH. For control and GSH-treated colonies, mitochondrion-rich regions were imaged as above for native fluorescence of NAD(P)H (excitation 365 nm, emission >420 nm), which is a general measure of redox state (Chance 1991). For control and GSH-treated colonies, using the Zeiss Axiovert 135 and an MTI CCD camera, 10-min videos of stolonal gastrovascular flow were taken at a point ~250 μm behind a peripheral stolon tip in five treated and five control colonies ~3 h after feeding. Three stolon tips per colony were filmed. Using Image-Pro Plus, 100 images of the videotape of each stolon were taken, one image every 5 s. Widths of both the stolon and lumen were measured for each image. Lumen width divided by cycle period and stolon width provides a proxy for flow rate (Blackstone 1998). While two-dimensional analysis of flow requires certain assumptions (e.g., Bagatto and Burggren 2006), these assumptions seem justified in this case.

Measures of Oxygen Uptake

In the first experiment, five treated and five control colonies were assayed using standard methods (Blackstone 2003). For a colony at 20.5°C, the rate of decline in oxygen concentration over a 30-min period was measured using a Strathkelvin 1302 electrode and a 781 oxygen meter. The chamber was then opened, a small volume of seawater was removed, an equivalent amount of the stock solution of GSH was added to achieve the target concentration of 65 μmol L^{-1}, the solution was mixed and aerated thoroughly with a small pipette, and the chamber was resealed. The rate of decline in oxygen concentration was then measured over another 30-min period. For the five control colonies, the same procedure was carried out, except no GSH was added to the chamber between the two measurements of oxygen uptake. Treated and control assays were paired in time. These assays were performed 3–5 h after the feeding of the subject colony as part of the normal feeding schedule. For each colony, the before/after difference in the rate of decline in oxygen concentration over a 30-min period was calculated. An overall trend in these differences for the five colonies was analyzed using a paired-comparison *t*-test. While efforts were made to insure that the colonies used in the experiment were as similar as possible, some differences were inevitable. The advantage of this experimental design is that each colony serves as its own control. Thus, the analysis focuses not on the absolute rates of oxygen uptake (e.g., mean rates for treated and control groups) but on how the treatment (or lack thereof) perturbed the oxygen uptake for each colony. The second experiment was carried out in a similar fashion but with a 2-h treatment between the first and second measure of oxygen uptake. Cysteine is capable of binding oxygen atoms, but at 65 μmol L^{-1} GSH, this was not detected using blank controls.

Results

Comparisons of Colony Growth and Development

Treatment of hydroid colonies with GSH (65 μmol L^{-1}) or GEE (1 μmol L^{-1}) triggers rapid growth of peripheral stolons away from the center of the colony, and the result is a fast-growing and runnerlike colony with few widely spaced polyps and long stolonal extensions (Fig. 1). Treated colonies all appeared completely healthy, and there were no indications of pathological growth. Higher concentrations of GSH (100 μmol L^{-1}) produce even more runnerlike growth forms, but colonies only survive for several days when treated at these higher concentrations. Such colonies do not appear unhealthy; rather, at 100 μmol L^{-1} GSH, the stolons simply fail to differentiate any polyps, and thus the colonies quickly starve. On the other hand, concentrations of GEE <1 μmol L^{-1} produced no effects. In these experiments, GEE was thus considerably less potent than GSH. From a cell-level perspective, this is surprising, because GEE enters cells much more effectively than GSH. While treated colonies were imaged at slightly smaller total areas than controls (mean ± SEM: GSH controls, 152.0 ± 10.9 mm^2, treated, 122.4 ± 8.8 mm^2; GEE controls, 163.9 ± 3.1 mm^2, treated, 106.3 ± 8.4 mm^2; GSH2 controls, 140.1 ± 10.2 mm^2, treated, 94.5 ± 5.9 mm^2), this likely reflects their runnerlike growth form. In other words, when contacting the edge of the cover slip, the long, unbranched stolons of the treated colonies enclosed a smaller total area than the more branched stolons of the controls (Fig. 1). In two of the three experiments, treated

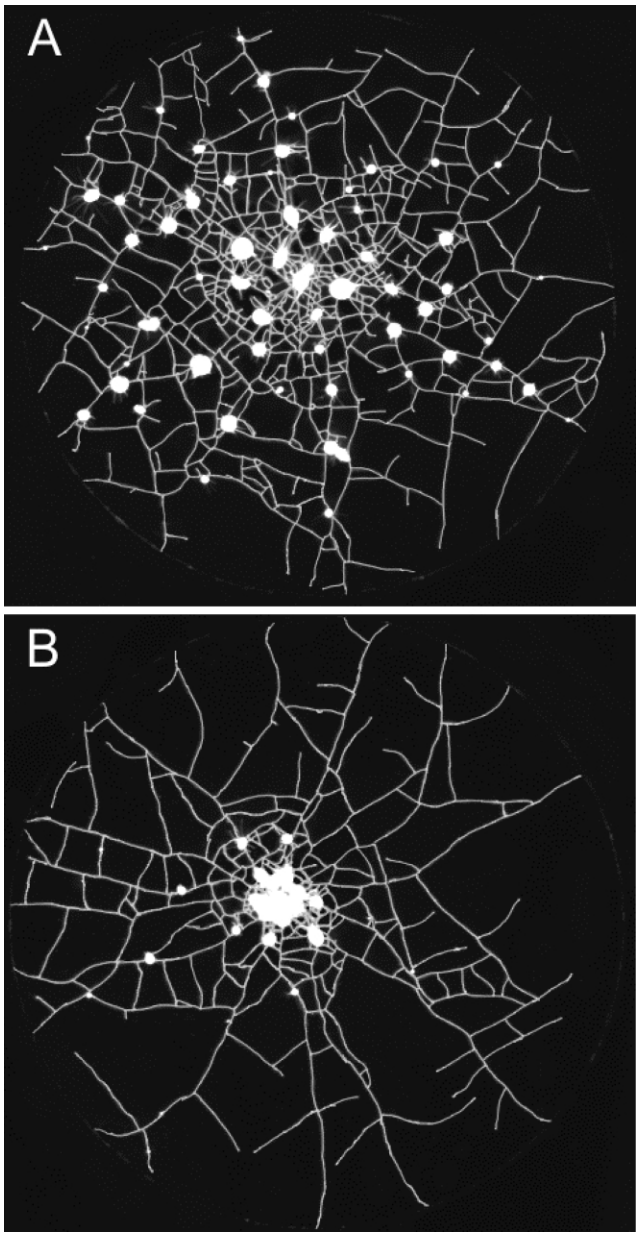

Figure 1. Processed images of genetically identical colonies of *Podocoryna carnea* growing on 18-mm-diameter glass cover slips near the time of covering the surface (*A*, control; *B*, treated with 65 μmol L^{-1} glutathione). Polyps are the brighter, circular areas, while stolons provide weblike connections.

colonies covered the surface in significantly less time than controls (GSH controls, 20.3 ± 1.1 d, treated, 19.8 ± 0.9 d; GEE controls, 30 ± 2 d, treated, 21.9 ± 1.2 d; GSH2 controls, 50 ± 6.3 d, treated, 30 ± 2.1 d). Variation among treatments is probably due to subtle seasonal or epigenetic effects (Ponczek and Blackstone 2001). Treated colonies exhibit less branching and anastomosis of stolons, as indicated by the mean size of unencrusted areas within the colony (Fig. 2; ANOVA of log-transformed data: GSH, $F = 10.9$, df = 1,12, $P < 0.01$; GEE, $F = 69.7$, df = 1,12, $P < 0.001$; GSH2, $F = 27$, df = 1,9, $P < 0.001$). Treated colonies also exhibited a smaller percent of the total area devoted to polyp growth (ANOVA of log-transformed data: GSH, $F = 18.7$, df = 1,12, $P < 0.001$; GEE, $F = 19.1$, df = 1,12, $P < 0.001$; GSH2, $F = 7.6$, df = 1,9, $P < 0.05$).

Comparisons of ROS, Redox State, and Gastrovascular Flow

Within 2 h, treatment with GSH or GEE shows an effect on peroxide and perhaps other ROS emitted from mitochondrion-rich cells (Fig. 3). Areas of H_2DCFDA-derived fluorescence detected automatically with Image-Pro software are either brighter (Fig. 3*A*) or larger (Fig. 3*B*) in treated colonies. Both differences

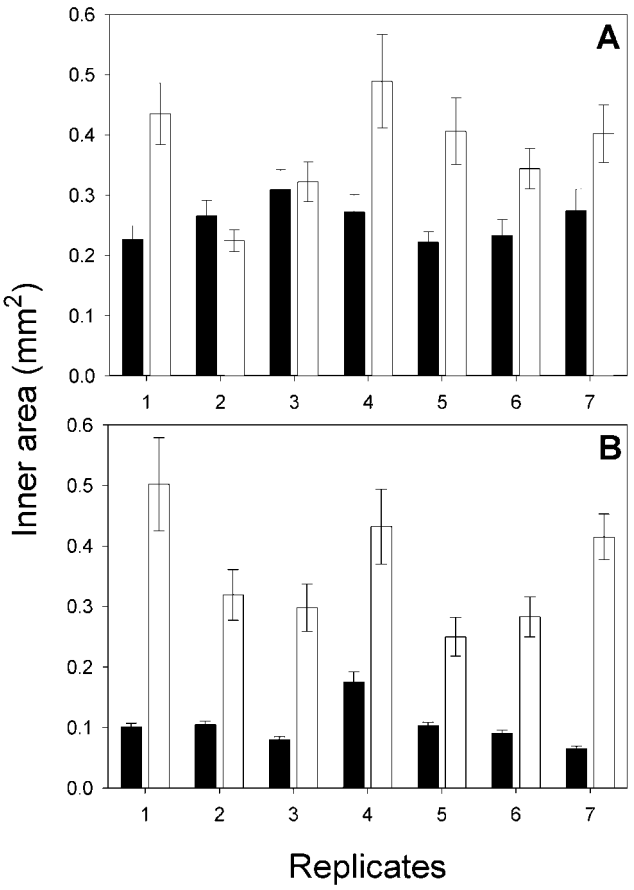

Figure 2. Treatment with glutathione (GSH) or glutathione ethyl ester (GEE) produces runnerlike colony growth forms. *A*, Mean ± SEM of the size of the areas of empty cover slip within the colonies ("inner area") for replicate control colonies (*filled bars*) and replicate colonies treated with 65 μmol L^{-1} GSH (*unfilled bars*). Each colony exhibits hundreds of inner areas. *B*, As in *A*, for colonies treated with 1 mmol L^{-1} GEE.

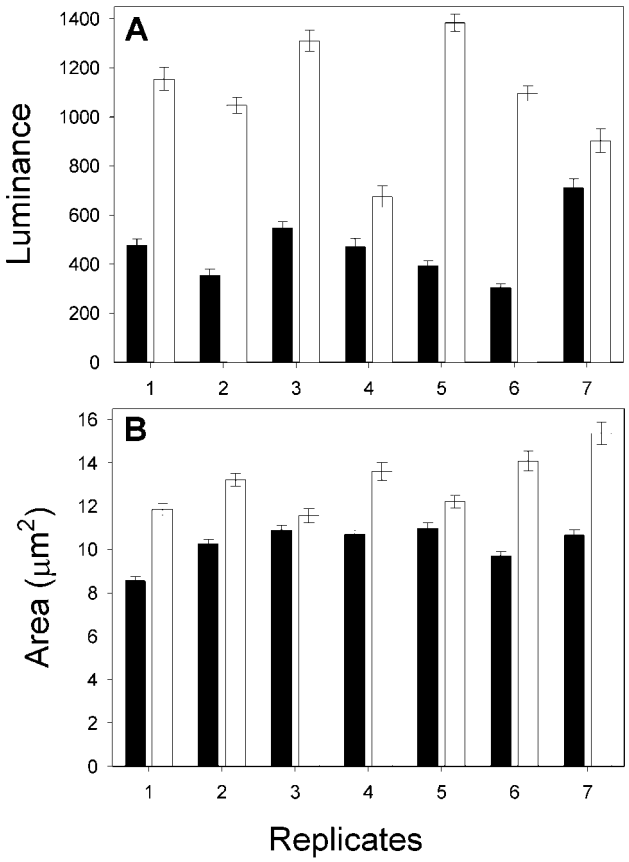

Figure 3. Treatment with glutathione (GSH) or glutathione ethyl ester (GEE) produces more reactive oxygen species from mitochondrion-rich epitheliomuscular cells at the base of polyps. Areas of H_2DCFDA-derived fluorescence detected automatically with Image-Pro software are either brighter (*A*) or larger (*B*) in treated colonies. *A*, Mean ± SEM luminance (foreground minus background fluorescence, grayscale from 0 to 4,095) for mitochondrion-rich cells from three polyp bases per replicate colony (*filled bars*, controls; *unfilled bars*, 65 μmol L^{-1} GSH). *B*, Mean ± SEM luminance area (μm^2) for mitochondrion-rich cells from three polyp bases per replicate colony (*filled bars*, controls; *unfilled bars*, 1 mmol L^{-1} GEE).

are significant (nested ANOVA: GSH, $F = 36.4$, df $= 1, 12$, $P < 0.001$; GEE, $F = 23.7$, df $= 1, 12$, $P < 0.001$).

The similar effects on colony growth, development, and ROS levels of GSH and GEE treatments suggest that both have the same extracellular effect. Subsequent experimentation focused on GSH, which exhibited greater potency. Treatment with GSH results in relatively reduced redox states of mitochondrion-rich cells in the base of polyps, as indicated by native fluorescence of NAD(P)H (Fig. 4; nested ANOVA: $F = 7.4$, df $= 1, 14$, $P < 0.05$). Peripheral stolon tips of colonies treated with GSH exhibit somewhat smaller amplitudes of opening and closing, and the period of opening and closing is increased as well (Fig. 5). Because stolon tips fill and empty largely in response to gastrovascular flow (Schierwater et al. 1992; Buss and Vaisnys 1993), GSH treatment thus produces diminished gastrovascular flow. Overall, a proxy for flow rate (lumen width divided by cycle period and stolon width) differs between control and treated colonies (nested ANOVA of arcsine-transformed data: $F = 28.6$, df $= 1, 8$, $P < 0.001$).

Measures of Oxygen Uptake

As described above, the experimental design focuses on whether the treatment (or lack thereof) perturbs the oxygen uptake of each colony. Treatment with GSH shows an immediate (within 10 min) increase of oxygen uptake (i.e., steeper oxygen vs. time slopes, Fig. 6*A*; paired-comparison *t*-test of five before and after slopes of oxygen concentration vs. time, $t = -8.81$, $P < 0.001$) compared with the controls ($t = -0.51$, $P > 0.6$). This effect was still present after 2 h of GSH treatment (treated colonies show an increase in oxygen uptake [Fig. 6*B*; $t = -5.8$, $P < 0.01$], while controls show a slight decrease [$t = 3.9$, $P < 0.05$]).

Discussion

Studies of redox signaling have been considerably illuminated by a bottom-up cell-level perspective. ROS have been identified as a frequent intermediary in redox signaling, and the genomic and proteomic targets of such signaling have in many cases been determined (Finkel 2006). In this regard, studies of chemical antioxidants are invaluable, and their predicted effects are usually clear a priori: within certain limits, antioxidants diminish ROS and thus can be used to experimentally decouple to some extent the effects of mitochondrial redox state and ROS. This is in contrast with studies that manipulate ROS levels

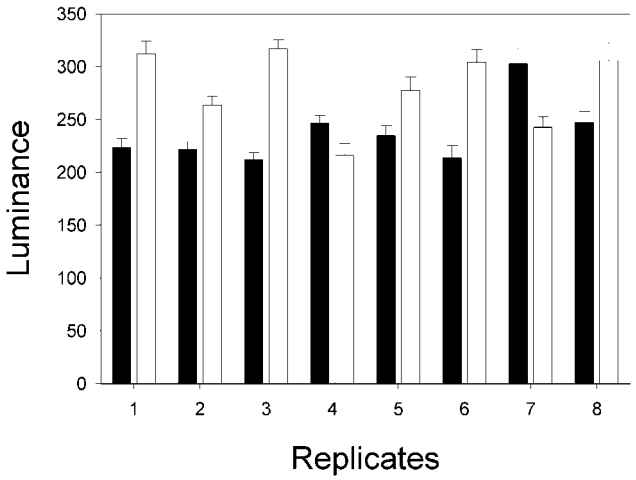

Figure 4. Treatment with glutathione (GSH) produces relatively reduced redox states. Native fluorescence of NAD(P)H is measured, and mean ± SEM luminance (grayscale from 0 to 4,095) is shown for mitochondrion-rich cells from three polyps per replicate colony (*filled bars*, controls; *unfilled bars*, 65 μmol L^{-1} GSH).

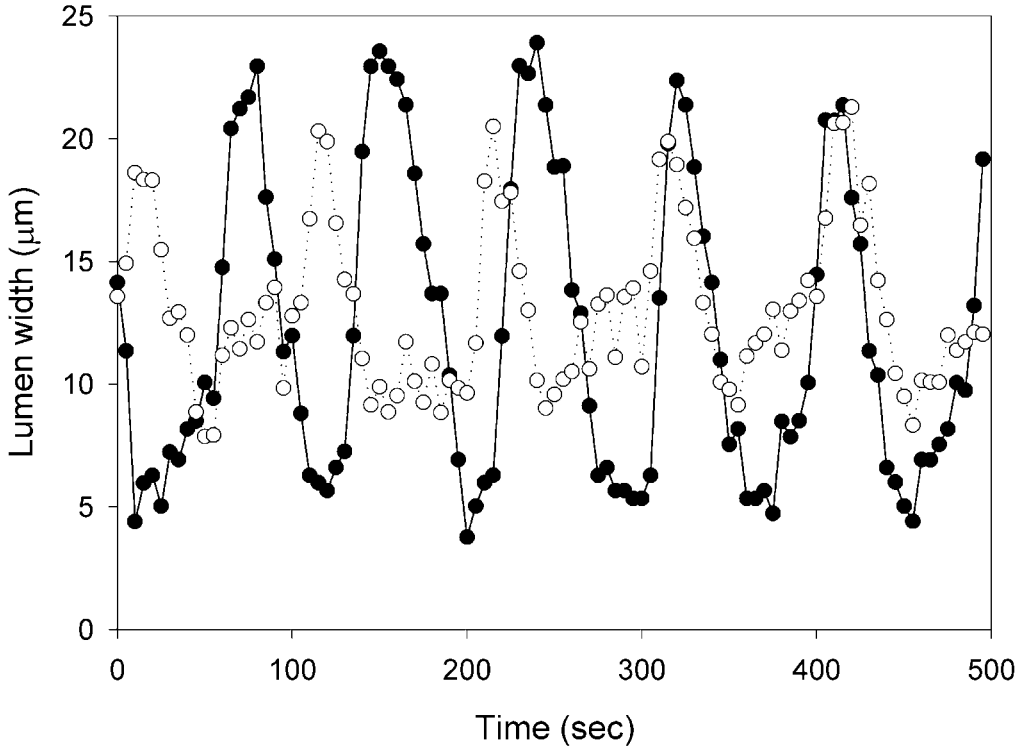

Figure 5. Glutathione (GSH) treatment diminishes gastrovascular flow. Oscillations in lumen width (μm) over time (s) for stolon tips of a control colony (*filled circles*) and a colony treated with 65 μmol L^{-1} GSH (*unfilled circles*). The treated colony shows lower amplitudes and longer periods of the oscillations; total stolon width was the same for both colonies.

by altering the mitochondrial redox state itself (e.g., Blackstone 2003). In other words, treatment with antioxidants typically will diminish ROS even if mitochondrial electron carriers are highly reduced and continue to produce quantities of these ROS. While to some extent this is an oversimplification (Lane 2002, 2005), it nonetheless serves as a useful starting point for investigations into the effects of antioxidants in vivo.

Relating this cell-level perspective to organism-level physiology often introduces greater complexity. In human plasma, for instance, Jones (2006) describes a surprising disequilibrium between the redox balance of GSH on one hand and cysteine on the other. These findings may be reconciled by recognizing that the balance between pro-oxidants and antioxidants is not defined by a single entity. While Jones (2006) redefines oxidative stress at both the cellular and extracellular levels, redox regulation at higher levels may in some cases merit separate consideration. The data presented on colonial hydroids are relevant here. A variety of environmental perturbations affect the growth and form of these colonies (Dudgeon and Buss 1996; Buss 2001; Blackstone 2003). At least in some hydroids, regions of mitochondrion-rich EMCs may have a central role in such colony-wide responses (Blackstone et al. 2004; Blackstone and Bridge 2005). The function of these mitochondrion-rich cells remains somewhat obscure, but in *Podocoryna carnea* their contractions probably open the gastrovascular lumen between the polyp and the stolon. Their actions can thus affect colony development both by their influence on gastrovascular flow and by their redox-related emissions, particularly of ROS.

Focusing on the latter, treatments of *P. carnea* colonies with GSH and GEE were carried out. From a cell-level perspective, the a priori predictions are clear: GEE enters cells and diminishes ROS with correlated effects on colony growth similar to the antioxidant vitamin C (Blackstone et al. 2005*a*), while GSH does not easily enter cells and thus has little or no effect. In contrast to these bottom-up predictions, both GSH and GEE had similar effects that contrast sharply with those of vitamin C: both triggered increased ROS emissions from mitochondrion-rich cells and correlated runnerlike growth. GSH produced these effects at considerably lower concentrations than GEE. Because the effects of GSH and GEE are not consistent with cell-level predictions, an organism-level top-down view of redox signaling (Blackstone 2006) is invoked to provide a hypothetical scenario consistent with these results. In turn, this scenario can suggest further experiments that may or may not provide additional support for the underlying hypothesis.

The effects of GSH and GEE on *P. carnea* may derive not from their antioxidant properties but from the GSH-mediated feeding response. This response may occur generally in cni-

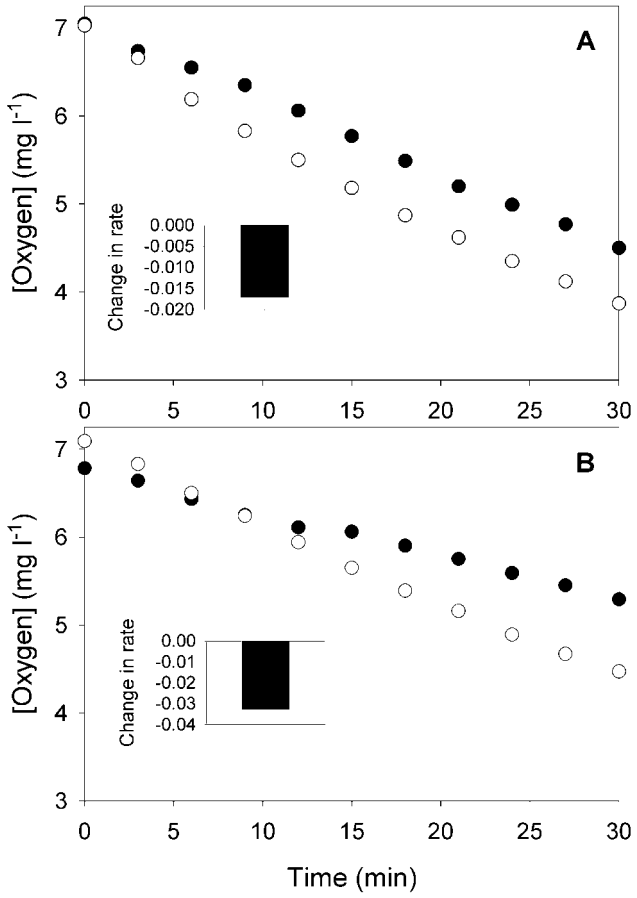

Figure 6. Rate of decline in oxygen concentration for a *Podocoryna carnea* colony before (*filled circles*) and after (*unfilled circles*) treatment with glutathione (GSH; 65 μmol L^{-1}). For five colonies of each treatment, inset plot shows the mean ± SEM of the before/after difference in the rate of decline in oxygen concentration where this decline is measured by the least squares slope of oxygen concentration versus time. This difference in rate was significantly negative; that is, the oxygen uptake increased after treatment. Oxygen uptake was measured in separate experiments immediately after treatment (*A*) and after 2 h of treatment (*B*).

darians and has been particularly well studied in *Hydra* species. In response to GSH concentrations on the order of 10 μmol L^{-1}, *Hydra* polyps writhe their hypostomes and tentacles and open their mouths (Lenhoff 1961). The response seems to be mediated by receptors for GSH and related molecules (Grosvenor et al. 1992) and can be prolonged via activation of putative glycine and GABA-like receptors (Pierobon et al. 2001). The ethyl ester tag may inhibit the binding of GEE to the GSH receptors, perhaps leading to the differences in effective concentrations of GSH and GEE. Observations of *Hydra* treated at 65 μmol L^{-1} also show a strong feeding response (K. Cherry-Vogt, unpublished data). Video microscopic observations of *P. carnea* treated at 65 μmol L^{-1} reveal that the hypostome and tentacles of treated polyps exhibit unusual jerking and twitching movements as compared with controls. These motions are consistent with the feeding response observed in *Hydra*. Unexpectedly, the feeding response may have a cascade of effects on colonies of *P. carnea* (Fig. 7). Oxygen uptake of the colony increases, probably because of the metabolic demands of the movements of the upper parts of the polyps (note that mitochondrion-rich cells have not been detected in these upper polyps; Blackstone et al. 2004). At the same time, because polyp-stolon junctions are typically closed during the feeding process (Dudgeon et al. 1999), contractions of the mitochondrion-rich cells in the polyp base probably decrease. The lumen connecting the polyps to the stolons thus remains largely closed, and gastrovascular flow diminishes. An increased fraction of the mitochondria of the mitochondrion-rich EMCs enter the resting state. The relatively reduced mitochondrial electron carriers of these cells are now more likely to donate electrons to oxygen. ROS formation thus increases, and a runnerlike growth form results.

While this scenario can account for the experimental data, clearly further tests of additional aspects of the overall hypothesis are needed, for instance, further exploration of the different effective concentrations of GSH and GEE. Related hypotheses such as a generalized stress response in the hydroids must be considered as well. In the event that further experimental work supports this scenario, some interesting implications deserve mention. Because of their disproportionate ef-

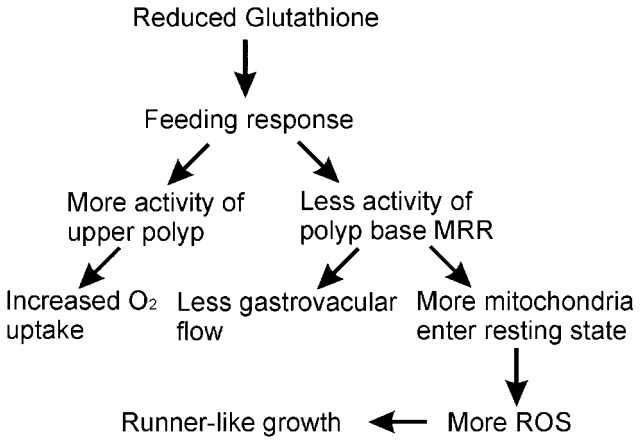

Figure 7. Schematic illustrating the hypothesized pathway of multicellular redox regulation of colonial hydroids treated with glutathione (GSH) or glutathione ethyl ester (GEE). GSH or GEE triggers feeding behavior of the polyps characterized by greater activity of the hypostome and tentacles. Oxygen uptake of the colony thus increases. At the same time, activity of the mitochondrion-rich epitheliomuscular cells in the base of the polyps decreases. These concentrations of cells serve as the multicellular redox regulator (MRR). Gastrovascular flow diminishes, and mitochondria of the MRR enter the resting state. Reactive oxygen species formation in the MRRs increases, and a runnerlike growth form results.

fect on the organism-wide redox signaling system, mitochondrion-rich contractile regions in the base of polyps could be viewed as "multicellular redox regulators" (Blackstone 2006). While this work focuses on cnidarians, multicellular redox regulation may be a common, though largely unrecognized, phenomenon in animals in general. Circulatory systems consisting of muscular, mitochondrion-rich contractile regions and fluid-carrying tubes are widely found in animals and may similarly drive organism-wide redox signaling. Similar processes may also occur in systems in which metabolic demand does not depend on feeding or muscular activity. The so-called mitochondrion-rich cells in fish gills (Hiroi et al. 2005; Tresguerres et al. 2006) provide a putative example, as do the cells of the salt gland in herring gulls (Chance et al. 1964) and other groups of cells involved in similar processes, for example, kidney tubule cells (Lane 2002). Ongoing genomic-level projects on hydroids and other cnidarians will facilitate examination of the molecular components of these processes. Clarifying the top-down pathways of multicellular redox regulation will be a major contribution of organismal biology to the emerging science of metabolomics.

Acknowledgments

Kim Cherry-Vogt provided invaluable help with the experiments and analyses. Several anonymous reviewers provided helpful comments. The National Science Foundation (grants IBN-00-90580 and EF-05-31654) provided support.

Literature Cited

Allen J.F., S. Puthiyaveetil, J. Ström, and C.A. Allen. 2005. Energy transduction anchors genes in organelles. BioEssays 27: 426–435.

Armstrong J.S., M. Whiteman, H. Yang, and D.P. Jones. 2004. The redox regulation of intermediary metabolism by a superoxide-aconitase rheostat. BioEssays 26:895–900.

Bagatto B. and W. Burggren. 2006. A three-dimensional functional assessment of heart and vessel development in the larva of the zebrafish (*Danio rerio*). Physiol Biochem Zool 79:194–201.

Balaban R.S., S. Nemoto, and T. Finkel. 2005. Mitochondria, oxidants, and aging. Cell 120:483–495.

Blackstone N.W. 1998. Morphological, physiological, and metabolic comparisons between runner-like and sheet-like inbred lines of a colonial hydroid. J Exp Biol 201:2821–2831.

———. 1999. Redox control in development and evolution: evidence from colonial hydroids. J Exp Biol 202:3541–3553.

———. 2001. Redox state, reactive oxygen species, and adaptive growth in colonial hydroids. J Exp Biol 204:1845–1853.

———. 2003. Redox signaling in the growth and development of colonial hydroids. J Exp Biol 206:651–658.

———. 2006. Multicellular redox regulation: integrating organismal biology and redox chemistry. BioEssays 28:72–77.

Blackstone N.W., M.J. Bivins, K.S. Cherry, R.E. Fletcher, and G.C. Geddes. 2005*a*. Redox signaling in colonial hydroids: many pathways for peroxide. J Exp Biol 208:383–390.

Blackstone N.W. and D.M. Bridge. 2005. Model systems for environmental signaling. Integr Comp Biol 45:605–614.

Blackstone N.W., K.S. Cherry, and S.L. Glockling. 2004. Structure and signaling in polyps of a colonial hydroid. Invertebr Biol 123:42–52.

Blackstone N.W., M.M. Kelly, V. Haridas, and J.U. Gutterman. 2005*b*. Mitochondria as integrators of information in an early-evolving animal: insights from a triterpenoid metabolite. Proc R Soc B 272:527–531.

Buss L.W. 2001. Growth by intussusception in hydractiniid hydroids. Pp. 3–26 in J.B.C. Jackson, S. Lidgard, and F.K. McKinney, eds. Evolutionary Patterns. University of Chicago Press, Chicago.

Buss L.W. and J.R. Vaisnys. 1993. Temperature induces dynamical chaos in a cnidarian gastrovascular system. Proc R Soc B 252:39–41.

Butterfield D.A., C.B. Pocernich, and J. Drake. 2002. Elevated glutathione as a strategy in Alzheimer's disease. Drug Dev Res 56:428–437.

Chance B. 1991. Optical method. Annu Rev Biophys Biophys Chem 20:1–28.

Chance B., C.-P. Lee, R. Oshino, and G.D.V. Van Rossum. 1964. Properties of mitochondria isolated from herring gull salt gland. Am J Physiol 206:461–468.

Chance B., H. Sies, and A. Boveris. 1979. Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527–605.

Coffman J.A., J.J. McCarthy, C. Dickey-Sims, and A.J. Robertson. 2004. Oral-aboral axis specification in the sea urchin embryo. II. Mitochondrial distribution and redox state contribute to establishing polarity in *Strongylocentrotus purpuratus*. Dev Biol 273:160–171.

Dudgeon S.R. and L.W. Buss. 1996. Growing with the flow: on the maintenance and malleability of colony form in the hydroid *Hydractinia*. Am Nat 147:667–691.

Dudgeon S.R., A. Wagner, J.R. Vaisnys, and L.W. Buss. 1999. Dynamics of gastrovascular circulation in the hydrozoan *Podocoryne carnea*: the 1-polyp case. Biol Bull 196:1–17.

Filomeni G., G. Rotilio, and M.R. Ciriolo. 2005. Disulfide relays and phosphorylation cascades: partners in redox-mediated signaling pathways. Cell Death Differ 12:1555–1563.

Finkel T. 2001. Reactive oxygen species and signal transduction. IUBMB Life 52:3–6.

———. 2006. A clean energy programme. Nature 444:151–152.

Gillette M.U. and T.J. Sejnowski. 2005. Biological clocks coordinately keep life on time. Science 309:1196–1198.

Grosvenor W., S.L. Bellis, G. Kass-Simon, and D.E. Rhoads. 1992. Chemoreception in hydra: specific binding of gluta-

thione to a membrane fraction. Biochim Biophys Acta 1117: 120–125.

Guarente L., and F. Picard. 2005. Calorie restriction: the SIR2 connection. Cell 120:473–482.

Haridas V., S.-O. Kim, G. Nishimura, A. Hausladen, J.S. Stamler, and J.U. Gutterman. 2005. Avicinylation (thioesterification): a protein modification that can regulate the response to oxidiative and nitrosative stress. Proc Natl Acad Sci USA 102:10088–10093.

Hiroi J., S.D. McCormick, R. Ohtani-Kaneko, and T. Kaneko. 2005. Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and CFTR anion channel. J Exp Biol 208:2023–2036.

Jefferies H., J. Coster, A. Khalil, J. Bot, R.D. McCauley, and J.C. Hall. 2003. Glutathione. Aust NZ J Surg 73:517–522.

Jones D.P. 2006. Redefining oxidative stress. Antioxid Redox Sig 8:1865–1879.

Lane N. 2002. Oxygen: The Molecule That Made the World. Oxford University Press, Oxford.

———. 2005. Power, Sex, Suicide: Mitochondria and the Meaning of Life. Oxford University Press, Oxford.

Lee J.-W. and J.D. Helmann. 2006. The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. Nature 440:363–367.

Lenhoff H.M. 1961. Activation of the feeding reflex in *Hydra littoralis*. I. Role played by reduced glutathione and quantitative assay of the feeding reflex. J Gen Physiol 45:331–344.

Nedelcu A.M., O. Marcu, and R.E. Michod. 2004. Sex as a response to oxidative stress: a twofold increase in cellular reactive oxygen species activates sex genes. Proc R Soc B 271: 1591–1596.

Nishikawa T., D. Edelstein, X.L. Du, S.-I. Yamagishi, T. Matsumura, Y. Kaneda, M.A. Yorek, et al. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787–790.

Pei Z.-M., Y. Murata, G. Benning, S. Thomine, B. Klüsener, G. J. Allen, E. Grill, and J.I. Schroeder. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406:731–734.

Pfannschmidt T., A. Nilsson, and J.F. Allen. 1999. Photosynthetic control of chloroplast gene expression. Nature 397: 625–628.

Pierobon P., R. Minei, P. Porcu, C. Sogliano, A. Tino, G. Marino, G. Biggio, and A. Concas. 2001. Putative glycine receptors in *Hydra*: a biochemical and behavioural study. Eur J Neurosci 14:1659–1666.

Ponczek L.M. and N.W. Blackstone. 2001. Effects of cloning rate on fitness-related traits in two marine hydroids. Biol Bull 201:76–83.

Schierwater B., B. Piekos, and L.W. Buss. 1992. Hydroid stolonal contractions mediated by contractile vacuoles. J Exp Biol 162:1–21.

Takano T., K. Sada, and H. Yamamura. 2002. The role of the protein tyrosine kinase Syk in oxidative stress signaling in B cells. Antioxid Redox Sig 4:533–550.

Tresguerres M., F. Katoh, E. Orr, S.K. Parks, and G.G. Goss. 2006. Chloride uptake and base secretion in freshwater fish: a transepithelial ion-transport metabolon? Physiol Biochem Zool 79:981–996.

van Montfort R.L.M., M. Congreve, D. Tisi, R. Carr, and H. Jhoti. 2003. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. Nature 423:773–777.